MEASURING THE EXPOSURE OF SONGBIRD NESTLINGS TO NEONICOTINOIDS IN NATURAL AND AGRICULTURAL NEST SITES

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Abstract

Neonicotinoid insecticides such as imidacloprid (IMI) have been under scrutiny for their damage on non-target invertebrates. However, recent literature suggests that grassland birds are experiencing population declines concurrently with increased IMI use. Field research was conducted to test the presence of IMI in Savannah Sparrow (SAVS) nestlings and their insect food supply at agricultural and non-agricultural nests. Using urine samples from Tree Swallows (TRES) dosed with IMI, an HP LC-MS was used to determine the concentration of IMI its metabolite 6-chloronicotinic acid (6-CN) at 0, 3 and 6 hours after exposure. Though no IMI or 6-CN residues were found in SAVS or their food, dosed TRES suggest birds can take upwards of 6 hours to excrete the parent compound and this was proportionate to the original dose. This will be a novel approach to neonicotinoid research on grassland birds and will serve as a guide to future research in this developing field.

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1. General Introduction

1.1 Grassland bird conservation

Over the past century, natural grasslands in North America have been immensely diminished in size. In fact, over 95% in area once covered by grasslands has disappeared (Samson and Knopf, 1994; Nocera and Koslowsky, 2011). As a result, grassland birds have begun using agricultural land as a replacement habitat (Hunter et al., 2001; Warren and Anderson, 2005; Nocera and Koslowsky, 2011) and these agro-ecosystems have become of great importance to biodiversity conservation efforts (Hunter et al., 2001; Freemark et al., 2002). The presence of birds in agroecosystems can be beneficial as they provide numerous ecosystem services in these environments (Sekercioglu, 2006; Wenny et al., 2011) such as seed dispersal, pollination, nutrient deposition and pest control (Wenny et al., 2011; Sekercioglu, 2006). In addition to the ecosystem services birds have an intrinsic value, they are a source of great cultural significance and are the most well documented and studied group of vertebrates (Wenny et al., 2011; Whelan et al., 2008; Sekercioglu, 2006).

In order to remediate current disturbances to ecosystems and prevent future bird population declines, it is important to identify the major causes behind grassland bird population loss. This decline in grassland birds is certainly due to habitat loss but the contribution to this decline of 'new generation' neonicotinoid pesticides is hotly debated (Hill et al., 2014; Mineau and Whiteside, 2013). Although Mineau and Whiteside (2013) suggested that pesticides like imidacloprid, one of the most common neonicotinoids, presented a greater threat to grassland birds than the expansion of cropland, a later study suggested that the

declines show a greater correlation with habitat loss than the toxic effects of the insecticide (Hill et al., 2014). It is likely there is an additive effect combining the stresses of habitat loss with increase vulnerability to insecticides (Mineau and Whiteside, 2013; Gibbons et al., 2015). There is clearly an urgent need to directly test exposure of birds to pesticides through field studies rather than broad correlative analyses.

1.2 Neonicotinoids

1.2.1 Development of neonicotinoids

In the early 1990s, concerns over anti-cholinesterase insecticides resulting in adverse health effects coupled with increased development of insect resistance set the stage for neonicotinoids, a new class of insecticides (Eskenazi et al., 1999; Morrissey et al., 2015). Imidacloprid was the first commercially available neonicotinoid, though from 2006 to present clothianidin has become more heavily used (Goulson, 2013). Neonicotinoids were the second most widely used agrochemical worldwide in 2015, the first being the herbicide glyphosate (Lopez-Anita et al., 2015). Its success triggered the development of multiple other neonicotinoids with similar chemical structures such as acetamiprid, clothianidin and thiamethoxamn (Jeschke et al., 2011). The increase in neonicotinoid diversity was followed by an increase in the diversity of crops treatable with this class of insecticide; 140 crops across 120 countries (Jeschke and Nauen, 2008). Neonicotinoids have been widely used in Ontario since 2004, primarily in the form of clothianodin and thiamethoxam (Schaafsma et al., 2016; Stewart and Baute, 2013). In 2013, up to 80% of soy, over 95% of corn and 100% of canola crop areas were planted using seeds treated with these neonicotinoids (Stewart and Baute, 2013).

The cause of this success lies in the many features that make neonicotinoids incredibly effective insecticides. Neonicotinoids work by binding to nicotinic acetylcholine receptors (nAChR), which play an integral role in the rapid excitatory synaptic transmission in the central nervous system (CNS) of insects (Jeschke et al., 2011). This ultimately over stimulates the nAChR causing paralysis and death in the invertebrate (Morrissey et al., 2015). However, despite this it remained an underexploited target site for insecticides for many years (Jeschke et al., 2011). Nicotine is a naturally occurring neurotoxin, some plants also use this target site as a defense against herbivory, however neonicotinoids have an enhanced selectivity for invertebrate nAChR than compared to nicotine alone (Sheets et al., 2016). As a result, neonicotinoids have a much lower toxicity to vertebrates, which increases the safety to non-target vertebrate organisms including agricultural workers (Sheets et al., 2016). Additionally, neonicotinoids are mostly applied as a seed coating, which reduces contact between workers while targeting the treated plant far more effectively compared to traditional methods of application such as foliar sprays (Jeschke et al., 2011). Despite this, neonicotinoids are also readily used in the form of foliar spray, soil treatments and in granular form for household pests (Goulson, 2013). Compounds such as 6-chloronicotinic acid (6CN), the major metabolite of the chloropyridinyl neonicotinoids such as imidacloprid, and 2-chloro-1,3-thiazole-5-carboxylic acid (2CTCA) the major metabolite of clothianidin and thiamethoxam have been documented in literature to measure the metabolism of these pesticides by humans and other mammals using urine samples (Nomura et al., 2013; Kavvalakis et al., 2013; Uroz et al., 2001).

The water solubility of neonictonoids facilitates their uptake into the plant body and distributes the pesticide from the roots to the leaves; this gives neonicotinoids their systemic quality (Goulson, 2013). This allows the insecticidal properties to protect the plant against a wide variety of herbivorous pests (Goulson, 2013). Neonicotinoids are effective in very small doses, they have an LD50 (lethal dose for 50% of individuals) that is 1/10 000th compared to historically used insecticides such as DDT (dichlorodiphenyltrichloroethane) but considered safer than organophosphates for toxicity to vertebrates (Goulson, 2013; Shukla et al., 2017).

The introduction of neonicotinoids within the agricultural sector as a commonly used insecticide was expected contribute to economic growth by dramatically reducing loss of crops, however this does not appear to be evident. Though crop yields have been growing since the second half of the 20th century, the introduction of neonicotinoids in the 1990s does not appear to coincide with any increase in yields (Goulson, 2013). In a comparison between treated and untreated soybean crops in the mid-south states in the US, on average it appears that there is a significant net economic return of $33USD/ha in treated sites (North et al., 2016). However, this average runs between 2005-2012 across Arkansas, Louisiana, Mississippi and Tennessee where only Louisiana and Mississippi experienced a significant return in the 7 year study period and only 4 of the 7 years had significant return between all states (North et al., 2016). Furthermore, almost all oilseed rape crops in the UK are treated with neonicotinoids, yet yields have not increased since its introduction (Goulson, 2013). The evidence suggesting that neonicotinoids are contributing significantly to economic growth is still inconclusive, raising the question of whether costs to the environment are indeed outweighed by gains to

society. It is critical to examine the efficacy of neonicotinoid pesticides and its potential impacts on non-target organisms to determine if its use is beneficial.

1.2.2 Neonicotinoids in the environment

The water solubility of neonicotinoids aid in rapid absorption by the plant, however it also enhances environmental persistence (Armburst and Peeler, 2002). Only about 5% of the active ingredient is absorbed by the plant itself, the rest remains in the environment (Goulson, 2014). A small portion is emitted in the form of dust and can be lethal to pollinators such as honeybees (Marzaro et al., 2011). The dust can also settle on non-target plants surrounding crop fields ultimately affecting many non-target insect species (Krupke et al., 2012). A vast majority of the active compound in seed coatings is leeched into the soil (Goulson, 2014). Due to a long persistence in the environment, soils in non-organic agricultural fields in France have been found to have concentrations of neonicotinoids such as imidacloprid upward of 1ppb (Bonmatin et al., 2005). Moreover, of the French agricultural fields tested, only 10 of the 62 positive sites had used neonicotinoids in the previous year (Bonmatin et al., 2005) which provides evidence for accumulation of these pesticides in soils, which can remain there for more than 2 years (Goulson, 2014). Though the concentration of 1ppb seems relatively insignificant, the LC50 for non-target insects and arthropods can be lower than this, therefore it can pose a high risk (Morrissey et al., 2015). Soil contamination means that root uptake by non-target plants can affect many non-target invertebrates (Krupke et al., 2012; Goulson, 2014).

Neonicotinoids can leach into groundwater and heavy rainfall can initiate runoff into streams carrying them into larger water bodies (Goulson, 2013). In a controlled experiment,

investigators simulated a heavy rainfall event on soil treated with a neonicotinoid and found 79% was readily leached from the soil (Gupta et al., 2008). Sampling of waterways such as rivers and creeks in California revealed that 89% of samples contained detectable levels of imidacloprid (Starner and Goh, 2012). Concentrations of neonicotinoids have been found to be as high as 225ppb of thiamethoxam in the playa wetlands of Texas, as well as 320ppb of imidacloprid in Dutch agricultural surface waters (Anderson et al., 2013; Van Dijk et al., 2013).

1.3 The effects of neonicotinoids on birds

1.3.1 Indirect effects

1.3.1.1 Reduction in food supply

Hallman et al.. (2014) used data from the Dutch Common Breeding Program (DCBP) from 2003-2010 for 15 passerine species and compared this with imidacloprid concentrations in nearby waterways. Most of the species were exclusive insectivores and all fed their young with invertebrates (Cramp and Perrins, 1994) so that any effect found could not be attributed to consumption of treated seeds. The researchers found a positive correlation between extent of bird population decline and imidacloprid concentration (Hallmann et al., 2014). Prior to the introduction of neonicotinoids in 1995, there was no evidence of declines.(Hallmann et al., 2014). Because of the dependence of these bird species on invertebrate food supply, the authors believed that food shortage due to the insecticide was likely explanation for their decline (Hallmann et al., 2014). Many aquatic invertebrates and insects with aquatic larval stages have also been in great decline, likely due to excessive pesticide use (Hallmann et al.,

2014). This study did not address any direct effects on the birds nesting in contaminated sites.

1.3.2 Direct effects

1.3.2.1 Reproductive success

Lopez-Anita et al. (2013) exposed red-legged partridges to seeds coated with imidacloprid using two treatments of high and low doses as well as a control group (Lopez-Anita et al., 2013). The low dose corresponded to the recommended applications rate according to Spanish regulations and the high dose was double the amount, meant to illustrate the effects of possible abuses of the product (Lopez-Anita et al., 2013). They discovered that egg shell thickness as well as egg length decreased significantly in the low dose treatment and that chick survival decreased for pairs exposed to imidacloprid, but unexpectedly this was not seen in the high dose treatment (Lopez-Anita et al., 2013). The high dose of imidacloprid resulted in a mortality of over 50% and it is possible that the remaining survivors had a natural resistance to the insecticide and so the sublethal effects were not observed (Lopez-Anita et al., 2013). The reduced eggshell thickness may be a result of the overall decreased body condition of the birds as the eggs had low amounts of protein, cholesterol, calcium and magnesium which mimicked the effects of starvation (Lopez-Anita et al., 2013).

In additional experiments it was found that imidacloprid treatment groups laid not only fewer eggs but the egg laying date was delayed compared to the control group (Lopez-Anita et al., 2015). Also, more vitamins and carotenoids were found in exposed eggs, but chicks did not have lower survival which the authors believed was due to the allocation of more resources for

a smaller clutch size (Lopez-Anita et al., 2015). The low mortality of the low dose group could also be due to the lower dose used than the previous investigation. Unlike the previous investigation, all birds in the high dose treatment were deceased within 21 days (Lopez-Anita et al., 2015).

Neonicotinoids can also have indirect effect on reproduction via muting secondary-sex traits, which in the red-legged partridge was tested with the eye-ring pigmentation (Lopez-Anita et al., 2015; Pérez-Rodríguez and Viñuela, 2008; Lopez-Anita et al., 2013). The intensity of pigmentation in the red-legged partridge is an indicator of the bird's health, since the pigment is derived from carotenoids from the diet, and both males and females select mates based on this colouration (Pérez-Rodríguez and Viñuela, 2008). Lopez-Anita *et al.* (2013, 2015) found that when exposed to low and high doses of imidacloprid the intensity of the red eye-ring pigmentation decreased significantly in both sexes compared to the control.

1.3.2.2 Growth and development

The thyroid plays an integral role in regulating the body's metabolic and endocrine functions, especially with respect to reproduction. Research on rodents noted thyroid disruption and thyroid lesions on individuals treated with an acute high dose of imidacloprid (Pandey and Mohanty, 2015; Zaror et al., 2010). Neonicotinoids have been reported to disrupt metabolic and reproductive functions in small mammals under laboratory conditions through the hypothalamic-pituitary-thyroid (HPT) axis (Anway et al., 2005; Bal et al., 2012; Bhaskar and Mohanty, 2014). Pandey and Mohanty (2015) examined the effects of imidacloprid on the

hypothalamic-pituitary-thyroid (HPT) axis in birds by exposing Red Munia (*Amandava amandava*), an Asian Finch species, to 0.5% of the LD50 of imidacloprid for a period of 30 days before and during the breeding phase. Thyroid hormones (T3 and T4) were measured during the preparatory or "pre-breeding" phase and the breeding phase in treatment and control birds. Though there was no significant loss in body weight of the birds exposed to imidacloprid, there was significantly lower T4 and TSH in both phases along with damage to thyroid follicles and lesions in the stroma. They concluded that the HPT axis of bird populations in the wild is very vulnerable to imidacloprid exposure even at current environmental concentrations (Pandey and Mohanty, 2015).

Thyroid hormones play a major role in regulatory processes, but also heavily affect reproductive success (McNabb, 2007). Thyroid hormones can impact gonadal development and egg-laying which can affect hatch date and eggshell formation; eggshell thinning was observed in imidacloprid treated birds in a previously mentioned study (McNabb, 2007; Lopez-Anita et al., 2013). Thyroid hormones also fluctuate throughout the day and night; disruption with this cycle can have an effect not only on foraging and breeding, but also on migration (McNabb, 2007). The effects of neonicotinoids on nestling development in wild songbirds has not yet been studied. This research would determine if these observed effects in laboratory experiments are effecting the reproductive and nestling success of wild grassland birds, and is critical to fill the knowledge gap on what is driving grassland bird decline.

1.3.2.3 Migratory ability

Migration is a very critical life stage for birds, there is an increased vulnerability due to the energy expenditure coupled with the potential consequences of delays in arrival to breeding grounds on reproductive success (Eng et al., 2017). A majority of migratory songbirds are unable to feed during the journey and rely on stores accumulated prior, sometimes doubling their body mass within a matter of two weeks (Bairlain, 2002). This rapid increase in food consumption can expose birds to agrochemicals such as neonicotinoids especially granivores likely to ingest coated seeds. Birds with longer migration trips are particularly vulnerable due to the higher amount of energy expenditure required to make the journey, making them more susceptible to neurotoxic insecticides like neonicotinoids (Eng et al., 2017). Researchers exposed wild caught granivore species (white crowned sparrows, *Zonotrichia leucophrys*) during Spring migration and exposed the adult birds to a control, high and low dose of imidacloprid (Eng et al., 2017). Birds were measured for body mass and migratory direction prior to dosing, dosed for three consecutive, measured after the dosing period and at 3 days and 14 days of recovery. Imidacloprid doses caused a significant loss in body mass after one exposure although body mass had recovered fully two weeks after treatment. The authors tested migration orientation of captive birds using outdoor 'Emlen funnels' to determine the direction birds oriented themselves based on environmental cues such as sunset and stars. Birds given the control dose maintained the orientation consistent with the pre-dose or baseline measurement but birds given the imidacloprid failed to orient themselves northward, or oriented the wrong way (Eng et al., 2017). Both low and high treated birds recovered to the

original direction after recovery. This study suggests that the assumption that imidacloprid has minimal direct effects on wild songbirds may be incorrect. Thus, investigating the exposure of wild grassland birds to these pesticides is integral to determine its potential role in population declines.

1.4 Research Objective

Given the documented sub-lethal direct effects of neonicotinoids on birds it is urgent to assess the extent to which wild populations are exposed, something that has rarely been done. Historical methods of determining neonicotinoid residue levels in birds consisted of highly invasive sampling of organs such as the liver and kidneys (Berny et al., 1999; Sauer et al., 2014). The goal of my study was to develop a less invasive sampling method based upon recent tests for neonicotinoid metabolite residue levels in mammalian urine using high pressure liquid chromatography mass spectrometry (HP LCMS) (Kavvalakis et al., 2013). These non-invasive sampling methods involve analyzing urine samples for traces of metabolites 6CN and 2CTCA. Urinary metabolites are biological markers that will allow us to determine not only exposure but also absorbed dose of an insecticide (Kapoor et al., 2014). Since completion of my research, new methods have been developed to use blood samples taken from White Crowned Sparrows, nevertheless, the question of whether urine tests can also be important for evaluation of exposure remains important (Hao et al., 2018). It would allow us not only determine the exposure but also the efficacy at which birds are able to metabolize it and excrete it, if any of the chemicals are potentially absorbed or retained.

To test the avian protocol for urine, it was predicted that there is a positive correlation between the amount of neonicotinoids a nestling birds were experimentally fed in a dosing study and the amount of residues found in the urine. Additionally, a negative correlation is predicted between the neonicotinoid concentration in the urine and the body mass of the nestling birds. This is because larger body mass is commonly used as a proxy for body condition, birds exposed to high concentrations will decline in body mass (Lopez-Anita et al., 2015). To test exposure of a wild grassland birds, it was predicted that both the insect food supply, and avian urine, would have a higher neonicotinoid contamination in intensive agricultural sites compared to birds nesting natural grasslands.

2. Materials and Methods

2.1 Study areas

2.1.1 Calibration Curve and Dosing study

Fecal samples were taken in Spring 2016 from nestling Tree Swallows (TRES) in nest boxes at Claireville Conservation which is a natural and protected grasslands under the authority of Toronto and Region Conservation Authority (TRCA). In Spring 2017, fecal samples were taken from nestlings at the Port Rowan Sewage Lagoon, part of the Long Point Bird Observatory (LPBO) TRES Project in Port Rowan, Ontario. The nestling dosing study was done at the Mud Creek (MC) colony site at Long Point because this site contained more active nests that were easily accessible. Though both sites did not have any pesticide use, both were in areas with large agricultural fields, the MC colony being adjacent to one.

2.1.2 Pesticide Exposure of Agricultural and non-agricultural nesting birds

To compare pesticide exposure of grassland birds nesting in natural and agricultural environments located in Guelph, Ontario samples were taken from Savannah Sparrow (SAVS) nestlings (Table 1). There were 6 non-agricultural (NA) sites and 8 agricultural (AG) sites which varied in size but were all at least 10 hectares. NA sites were grasslands not being used for agriculture while AG sites were farms that supported various row crops such as corn, soy and wheat. Most NA sites were historically, at least five years ago, used for agriculture but are now restored grasslands in conservation areas. The other NA sites were grassy areas with low

disturbance. Farmers of the AG sites were not asked about pesticide use, however site KEAG was a transparently an organic farm.

2.2 Study design

2.2.1 Urine extraction protocol

To collect urine, all nestlings were removed from a nest box and individually placed in plastic cups. Once a sample was produced, it was collected from the cup using a Pasteur pipette and placed into a microtube which was stored in a cooler. Birds were not kept in the cups for longer than 5 minutes to prevent stressing. The birds were not weighed or handled any more than necessary to take a sample. The ages of the birds used for blank samples varied but were at least 7 days old (nestlings fledge around 14d). All birds from a given nest box were returned to the nest at one time and at least one hour was given before the same nest box was sampled again. Cups were sprayed with ethanol and wiped between sampling. Once a sufficient number of samples were collected, the samples were taken back to the lab and placed in the -20C freezer.

To test the urine of birds for traces of Imidacloprid metabolite 6CN and the Clothianodin and Thiamethoxam metabolite 2CTCA, an avian protocol was adapted from the mammalian protocol of Kavvalakis et al. (2013). The mammalian protocol was used for rabbits and humans, thus the working volumes of urine were far greater per individual than could be obtained for small songbirds The working volumes for birds were therefore reduced to 2% to accommodate the low volume of urine from birds (Figure 1). Nestling birds were used rather than adults due

to the presence of fecal sacs facilitating the collection of urine in nestlings, since the fecal sac acts as a membrane keeping the urine contained.

2.2.2 Calibration curve

Fecal samples from these 2 sites were used as blank urine samples to create a calibration curve. To create a calibration curve, blank urine was spiked with various concentrations of the metabolites 6CN and 2CTCA ranging from 0 to 25ug ml-1 (0, 0.1, 1, 10 and 25ug ml-1) in 2016. When samples were ready for the extraction protocol outlined in Kavvalakis et al. (2014), the samples were thawed at room temperature and subsequently kept on ice. Samples were placed in the centrifuge at 14000 RPM for 10 minutes at 4C to facilitate pipetting out the liquid urine from the feces and uric acid. The blank urine was pooled in 2016 and 2017 with the samples collected the same year and vortexed to mix. The pooled urine was divided into several 50uL samples and spiked with concentrations (Table 2). Spiked samples were stored in the -20C and taken to AFL on dry ice and analyzed use HP LC-MS.

In 2017, this was repeated with the exception of the final step of extraction. Unlike the protocol followed in 2016, samples were not evaporated using a nitrogen stream but left overnight in the fume hood to evaporate. This was due to a lack of access to appropriate equipment.

2.2.3 Dosing study

Prior to field work, the imidacloprid doses were made for the dosing study designed to validate the urine protocol. There were 3 treatments: control, low and high doses. The low and

high doses were based off the of the LD50 of House Sparrows (*Passer domesticus*), at 2.5% (1.025ug/g body weight) and 10% (4.1ug/g bodyweight) respectively (Eng et al.. 2017). The vehicle for the doses was certified organic sunflower oil, therefore the control was the vehicle alone. Doses the volume of 10ul/g bodyweight (bw) was to be administered to each bird. Using the typical weight of a TRES nestling at least 7 days old, the stock volume required for each treatment was calculated.

Stock volume = (mass of bird) X (volume administered) X (birds treated)
Stock volume ≅ (20g) X (10µl/g bw) X (5 birds × 30 nests) ≅ 30 000µl OR 30ml

To ensure there was enough stock solution, twice the required volume (60ml) was created. Imidacloprid is UV-light sensitive, so all stock solution was stored in amber bottles. To create the solution, the required amount of imidacloprid was weighed into an amber bottle and dissolved using a small volume of acetone, such as 500ul. The final volume of sunflower oil was added to the bottle which was then placed on a stir plate and loosely covered with tin foil. The solution was left to stir overnight so that the acetone could evaporate out of the mixture.

Data on hatch date of each nest (obtained from Long Point Bird Observatory staff) was used to determine the age of nestlings, and only nestlings at least 7 d old were included in the dosing experiment . Each of the 3 treatments was run on 10 nests for a total of 30 nests used in the experiment. Nestlings were weighed at 0, 3 and 6hrs after dosing at which time fecal samples were also collected. Nestlings were taken from the nest box at one time and placed in individual plastic cups to defecate and samples were collected with a Pasteur pipette. Nestlings did sometimes excrete in the nest prior to being placed in a cup, and if easily accessed the

sample was collected and placed in a vial. All sample vials collected at a given sampling time from a nest were added in a bag labelled with the nest ID and the time of sampling. After defecating, the nestlings were individually weighed and identified using either a pre-existing leg band number or from magic markers on their leg(s). Using this identification, the weight of each nestling could be tracked.

After the fecal samples and body mass was collected for time 0, each nestling received a dose based on their weight. The weight of the nestling was rounded to the nearest gram so an approximate dose could be measured. For example, a nestling weighing 19.8g was given a dose of 120ul or 0.12ml instead of 0.198ml due to the graduations on the syringes. For access to small volumes of the doses during field work, a 2ml microtubes covered in tin foil was filled with the respective dose for each nest. To administer a dose, plastic 1ml syringes with 10ul graduations and plastic, gavage feeding needles with silicone tips were used as per Eng et al. (2017). The dose was extracted from the working volumes in the microtube prepared for the nest based on the weight of the nestling. The silicone tip of the plastic needle allowed a comfortable insertion into the beak and down the esophagus. The tip of the gavage needle could be felt once fully inserted into the crop of the bird, the dose was ejected from the needle and carefully pulled out. Each needle assembly was used for all the nestlings in a nest and disposed.

After given a dose, each nestling was returned to the nest and the nestlings were revisited after 3 and 6 hours of their dose, fecal sampled and then weighed and held for no longer than 5 minutes. If no sample was provided, the nestling was weighed and returned to

the nest. Samples collected were placed on ice packs in a cooler and at the end of the field day, the samples were placed in a -20°C freezer.

The pooled samples were vortexed to mix and 50 µl was taken from every pooled sample and run through the extraction protocol. Samples were pooled based on nest and time. The extracted samples were sent to the Agricultural Food Lab (AFL) in Guelph, ON for HPLC MS to determine levels of 6CN.

2.2.4 Exposure of wild grassland bird population

Nest searching began in May for SAVS nesting in non-agricultural grasslands and the grassy laneways of agricultural field. Nests were numbered for each site and field work was conducted MSc student Heidi van Vliet's for her research on nesting success. The urine samples were collected from 6-9 day old nestlings at the time of banding, using the same methods as described above. The). In 2016, the samples were separated based on individual and 50ul of urine was taken from each sample. In Spring 2017, urine samples from the same nest were pooled in lab. Pooled samples were vortexed and up to 4 x 50ul samples were taken from each sample. Each 50ul sample from 2016 and 2017 was run through the extraction protocol and sent to AFL in for HPLC MS to determine levels of 6CN and 2CTCA.

Insect samples were also collected to determine potential food source exposure to the pesticides. To obtain this sample, a sweep net was used for 3x25m transects or 4x25m transects if the sample was not large enough. The contents of the net were placed into a sealed bag and placed in a -20C freezer. In the lab, the bag was placed on ice and the insects were collected and placed into 50mL plastic conical tubes while plant matter was removed. The insect tubes

were placed in the -20C freezer and transported to AFL for 6CN and 2CTCA analysis using HPLC MS.

2.3 Statistical Analysis

2.3.1 Dosing study

We tested for differences in detection of neonicotinoids in TRES nestlings for different treatments over time. We fitted a generalized linear mixed model (function glmer, package lme4 in R) with treatment and time as predictor variables and detection as a response. Nest ID was set as a random effect to account for independent differences between nests. Models were fit with binomial distribution based on presence/absence of neonicotinoid residues in urine used chi-squared tests to determine if treatment and time after dose were significantly different. Post-hoc comparisons were conducted on significantly different factors using multiple pair wise comparisons (function glht, package multcomp). These statistical analyses were conducted in R 3.5.0. Differences in body mass of TRES nestlings at different treatments over time were tested using Kruskal-Wallis one-way ANOVA on ranks with SigmaPlot (Systat Software, San Jose, CA). We conducted post-hoc comparisons on significant different factors using non-parametric multiple pairwise comparisons Dunn's Method.

2.3.2 Calibration Curves

Calibration curves were tested using a multiple linear regression analysis on SigmaPlot (Systat Software, San Jose, CA) in 2016 and a linear regression for 2017 data. For between metabolite comparisons of significance, data were tested using ANCOVA on SPSS (IBM).

3. Results

3.1 Calibration Curves

The calibration curves created in 2016 and 2017 represent different spike concentrations but ultimately generate the same linear trend, an increase in spike concentration (ug ml-1) of the pesticide metabolites results in a higher recovered amount (ppm; Figure 4). In 2016, the spike concentrations ranged from 0 to 25ug ml-1 of both IMI metabolite 6CN and clothianidin metabolite 2CTCA while the 2017 spikes focused on the smaller range of 0 to 2ug ml-1 for only 6CN (Figure 4). The highest recovered amount of 2CTCA resulted from the 25ug ml-1 spike, 2.6ug ml-1 was recovered after following the extraction protocol and analyzing the samples using HP-LCMS (Figure 4). The highest recovered amount of 6CN recovered (4.3ug ml-1) also resulted from the 25ug ml-1 spike but was greater than what was found for 2CTCA (Figure 4). There was a significant multiple linear regression [$F(2,2)=976.602$, $p=0.001$] for 6CN ($n= 5$) and 2CTCA ($n= 5$) with an R^2 of 0.999 in 2016, and linear regression of 6CN ($n= 5$) in 2017 ($p= 0.018$) with an R^2 of 0.94.

3.2 Dosing Study

A positive trend was observed between dose and the amount of detected IMI excreted over time in TRES nestlings for both the high and low treatments (Figure 2). All three treatments had no IMI detected in samples taken prior to the dosing. The highest mean IMI detected was at 6hrs post-dose in the high treatment, $1.18 \times 10^{-3} \pm 3.84 \times 10^{-4}$. Treatment was highly significantly correlated to the presence of neonicotinoid residues in urine samples

[$\chi^2(2)=13.9067$, $p=0.0009$] under a generalized linear model. The proportion of nestling samples with detected quantities of IMI were significantly greater in the high treatment compared to the control ($p=0.0159$; Figure 2). The proportion of samples with detected quantities of IMI for nestlings fed the low treatment was not significantly different from the control or the high treatment ($p=0.4623$ and $p=0.0816$ respectively). The proportion of samples with detectable IMI increased in both the high and low treatment between 3 to 6hrs post dose (Figure 2).

The average loss in body mass (g) over time after a dose of IMI showed similar trends in all three treatments (Figure 3) with a steeper decline in body mass during the first 3 hours than the final 3 hours of the experiment. Average mass loss after 6hrs represented about 3% of average starting body mass (21.5g ± 0.17) for the low treatment. Unexpectedly, nestlings in the low dose treatment lost significantly more body mass than nestling in the high treatment [$\chi^2(2)=7.311$, $p=0.026$] only at 6hrs after exposure (Figure 3).

3.3 Exposure of wild grassland population

Insect samples taken near SAVS nests in 2016 and 2017 were tested for presence of neonicotinoid metabolites 2CTCA and 6CN along with IMI. In 2016, 4 of 16 sites with insect samples had trace amounts of 2CTCA but in 2017 0 of 16 samples contained this metabolite. Of these 4 sites, 3 were found to have traces below the detection limit of 0.002ug ml-1 (ASNA6, CLAG3 and STAG; Table 1) and which were non-agricultural (ASNA6) and agricultural (CLAG3 and STAG). The other site, GUAG5, did have residues above the detection limit but below the quantification limit of 0.005ug ml-1 and was estimated to be 0.0024ug ml-1. No insect samples

had traces of IMI or its metabolite 6CN during this investigation in 2016 or 2017. Soil samples collected in 2016 also found no residues of IMI, 6CN or 2CTCA (sampling was not conducted in 2017).

Nestling urine samples collected from 16 SAVS nests in 2016 did not contain any residues of the metabolites 6CN or 2CTCA. However, potential trace amounts of IMI were found at nest ASNA2, a non-agricultural site, which was below the limit of detection (LOD) of 0.0008ug ml-1, while confirmed residues above the LOD were found at nest HCNA3 (non-agricultural) estimated to be 0.0012ug ml-1. The exact amount cannot be confirmed because it was below the limit of quantification (LOQ) of 0.0025ug ml-1. In 2017, no neonicotinoid residues were found at any of the 8 sites observed. Although the ASNA site was studied in both 2016 and 2017 residues were found in only the first year, and the HCNA site which had confirmed residues of IMI in 2016 did not produce successful nests for sampling in 2017 (Table 1).

4. Discussion

4.1 Dosing Study

The first prediction was that if the protocol is effective, and if birds excreted neonicotinoids similarly to what has been seen in mammals, then we can expect the residues found in their urine to correspond with the dose given. The significant linear regression on both 6CN and 2CTCA in 2016 and 6CN in 2017 means that the urine extraction protocol allows for a recovery of residues that leaves a predictable original concentration of the neonicotinoid metabolite(s) in the urine.

While it is not conclusive, the findings from the TRES experiment show mean IMI recovered from the urine of dosed nestlings was higher than controls. In previous research it was observed that female rat peak residues can be found at 12hrs after exposure (Kapour et al., 2014) but small birds have a higher metabolic rate and were expected to peak within 6 hours. The mean IMI recovered was not statistically significant between dose and time after dose, but the strong effects size suggests low statistical power due to modest sample sizes. The number of samples with confirmed presence of IMI residues (above the minimum detection limit) was significantly higher in high dose birds compared to the control. This suggests that the amount of exposure does affect the presence of IMI residues in bird urine, but further study is needed to assess IMI quantity.

Eng et al. (2017) found 10% LD50 dose adult White-crowned Sparrows experienced a 17% decline in body mass after 3 consecutive days of dosing. Thus, one might expect Body mass of nestling TRESs after dosing to decrease with higher exposure to IMI , however it was the low

dose birds who lost mass most rapidly. In partridges, Lopez-Anita et al. (2013) also found low dose treatment birds exhibited greater reproductive losses after exposure than the high dose and attributed this to the initial high mortality rate in high dose birds it and more resilience to IMI in the survivors (Lopez-Anita et al., 2013). For this investigation there was no observed mortality in TRES nestlings administered any dose. The number of doses, and the short 6hr experimental window for this experiment on nestlings may have been too narrow to observe the potential effects on body mass by a single dose. The TRES nestlings weigh on average 21.5g, so a 0.6g or below 3% loss in body mass may be biologically irrelevant and can be attributed to non-experimental factors.

4.2 Exposure of wild grassland birds

4.2.1 Food source contamination

It was predicted that insects in agricultural sites would contain greater amounts of neonicotinoid residues compared to insects in natural grasslands due to the historic presence of neonicotinoids in Southern Ontario farms (Schaafsma et al., 2016). However, only 1 insect sample collected over 2 years contained a trace of neonicotinoids above the minimum detection limit and no soil samples from 2016 were contaminated. Birds in agricultural sites nested beside row crops of corn and soybean, that most likely were treated with neonicotinoids in recent years, and adults were frequently observed foraging on the ground in the fields. At least in June and July, when samples were taken, there is no evidence birds were being exposed to neonicotinoids through food or soil.

4.2.2 Nestling urine samples

It was predicted SAVS nestlings in agricultural sites would face greater exposure to neonicotinoids and have residues present in their urine. However, no traces of neonicotinoids were found in nestlings in either agricultural or natural sites. This suggests that if birds are exposed to neonicotinoids, it may be consumed and excreted at such low levels that it cannot be detected by common HPLC-MS methods. New methods have recently been developed to use non-lethal blood samples from migratory White Crowned Sparrows to determine exposure and is more sensitive to very low levels (parts per trillion) of multiple neonicotinoids found present in dosed and wild caught birds samples (Hao et al., 2018). These new methods may be critical for the next steps in this research (Hao et al., 2018). Given the LC50 of House Sparrows (41ug/g bw) the results of this investigation suggest that these very low levels are not a threat to SAVS survival or reproduction (Eng et al., 2017). Nestlings were sampled once after 7 days of potential exposure after hatching, and the food sampling showed little to no contamination. Thus it seems reasonable to conclude that neonicotinoids are not a threat to these nestlings, despite nests being in close proximity to crops where neonicotinoids have been widely used. It is possible that adults face higher exposure, and threats, if they encounter and eat treated seeds when they arrive in spring. To further verify these results, it is necessary to repeat the experiment with increased sampling of nestling at both natural and agricultural sites.

Similarly, residues of neonicotinoids in low levels were found in blood samples taken from Honey Buzzard nestlings with nest sites near oilseed plant agricultural fields (Byholm et al., 2018). Honey Buzzards feed primarily on insects especially as nestlings so the route of

exposure is to potential neonicotinoids is likely oral as it is for SAVS nestlings (Byholm et al., 2018; Itamies and Mikkola, 1972). Now that blood sampling can reveal exposure to even tiny amounts of neonicotinoids, it will be possible to test for adult exposure repeatedly over the breeding, to better compare agricultural and natural sites, and to test if exposure predicts body condition and nesting success. Although the present study did not find evidence that neonicotinoids are a threat to nestling SAVS, it would be premature to conclude that these pesticides are not linked in some way to grassland bird declines.

4.3 Conclusions and Future Research

The objective of this research was to determine a novel non-invasive sampling method to test grassland birds for exposure to neonicotinoids. Previous research has suggested that exposure to these pesticides can have detrimental effects on birds indirectly and directly (Lopez-Anita et al., 2015; Hallmann et al., 2014; Gibbons et al., 2015; Eng et al., 2017). As populations declines of grassland birds continue across North America it is imperative to further develop these methods to better determine the potential impact neonicotinoids are having on these populations (Eng et al., 2017; Mineau and Whiteside, 2013). Recent research on highly sensitive methods using blood plasma has revealed the presence of neonicotinoid residues circulating within wild caught migratory songbirds which suggests that non-dosed birds are exposed to enough of the compound for its routine detection (Hao et al., 2018). Although using these non-invasive blood samples in future research could be greatly beneficial, blood samples are limited by body size which may be problematic for testing nestling birds and the overall

metabolism of these compounds (Hao et al., 2018). Additional research has revealed simpler, more non-invasive feather samples taken from House Sparrows can detect even higher levels of various neonicotinoids even on organic farms (Humann-guilleminot et al., 2019). However, this can only be used for research regarding potential exposure, not regarding concentrations present in the body. Further development of urine samples specific to avian urine still aid in testing for exposure and also for metabolism over time as more samples can be taken at shorter time intervals. Although it is not conclusive, this research suggests that nestling birds even with high metabolic rates may take more than 6 hours to remove neonicotinoids from their system. Further understanding of avian metabolism of these compounds is vital to new ecotoxicological research and understanding the population decline of grassland birds.

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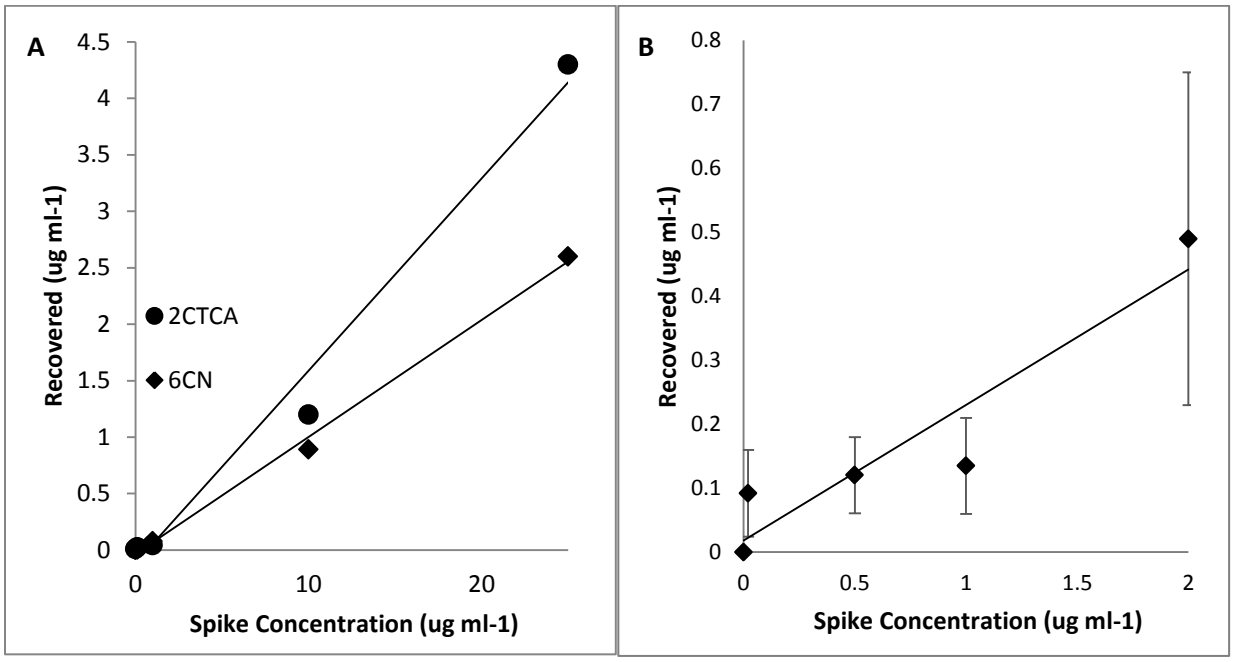

Figure 1: Calibration curve for spiked Tree Swallow nestling urine samples taken in 2016 at (a) Claireville Conservation Area (n= 14 samples) and (b) 2017 at Long Point Bird Observatory (n= 10 samples). Note the different x-axis scales. There is no significant difference between 6CN and 2CTCA in 2016 ($p=0.179$), there is a very significant correlation between the spike and recovery ($p=0.001$). Similarly, in 2017, there is a significant recovery of mean spiked concentrations of 6CN ($p=0.018$).

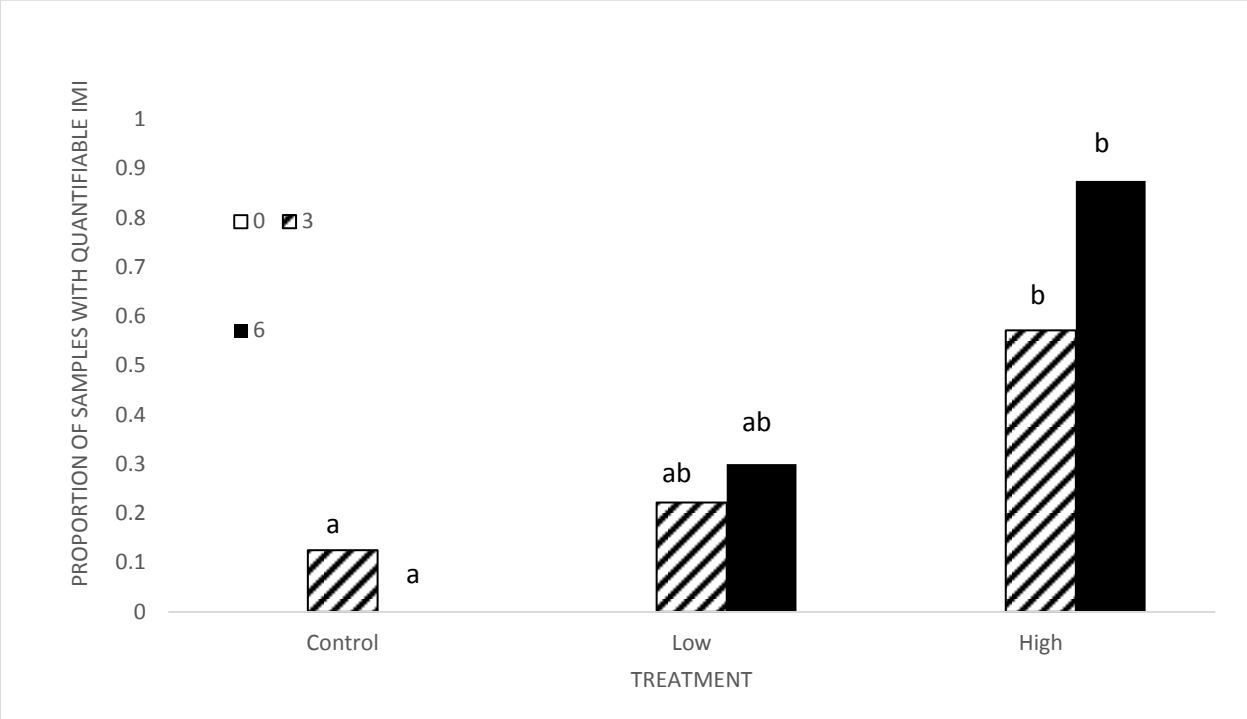

Figure 2: Proportion of Tree Swallow nestling urine samples collected with quantifiable amounts of IMI under control (n= 24 samples), low (n= 28 samples) and high (n= 24 samples) dosing treatments at 0, 3 and 6 hours after exposure. No samples had quantifiable levels of imidacloprid at time 0 hours, thus the bar is not present on the graph. The high treatment had a significantly greater proportion of quantifiable samples than the control at both time 3 and 6 hour samples ($p<0.05$) as indicated by the lower case letters.

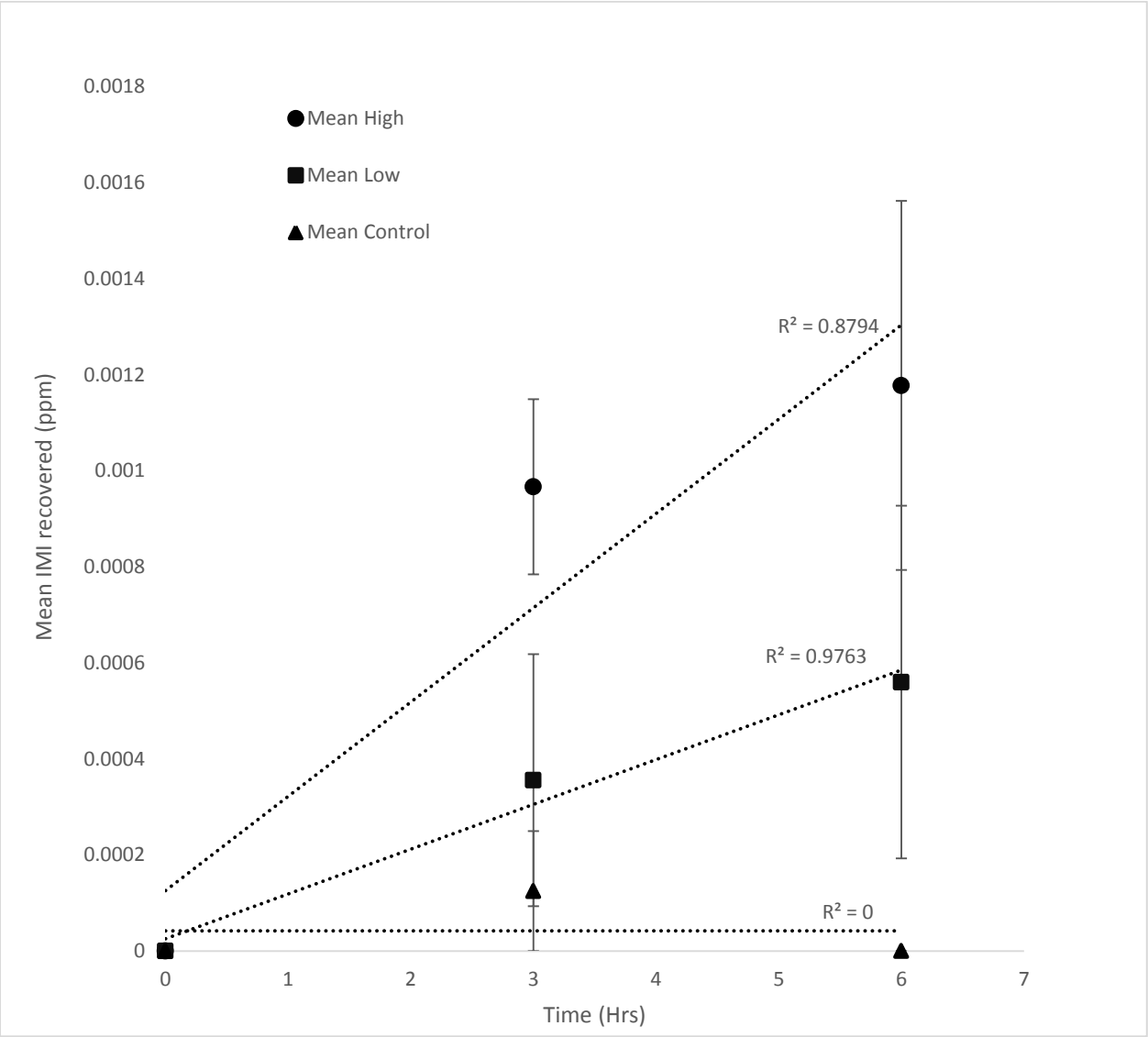

Figure 3: Mean (+/- SE) 6CN detected in Tree Swallow pooled nestling urine samples a for the high (n= 24 samples), low (n= 28 samples) and control (n= 24 samples) treatment at each sampling time (0, 3, and 6 hours after dosing).

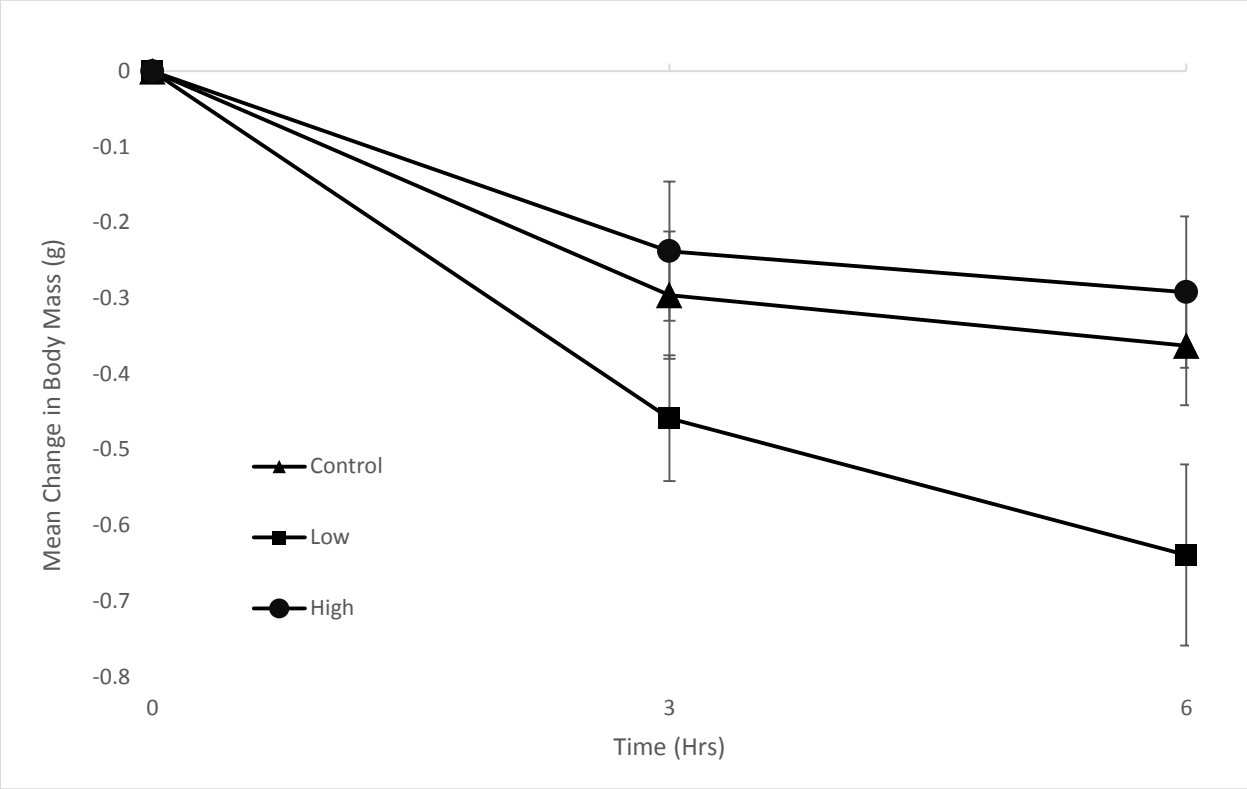

Figure 4: The mean change in body weight of Tree Swallow nestlings for each treatment over the sampling period of 6hrs. There is a significant difference between the high (n= 42 individuals) and low (n= 41 individuals) treatment at 6hrs after the dose (p=0.026), but neither is significantly different than the control (n= 48 individuals). Average body mass of 21.5g ± 0.17 at time 0hrs.

Table 1: Sites used for Savannah sparrow study in Guelph, Ontario and samples collected in 2016 (n= 22 individual urine samples) and 2017 (n= 65 pooled urine samples).

Years	Site ID	Size (Hectares)	Site Type	Total # of nests found	Total # of fecal samples	Total # insect samples
2016-2017	ASNA	16.4	Non-agricultural	17	15	4
2016-2017	GLNA	28.7	Non-agricultural	13	16	3
2016	HCNA	11.3	Non-agricultural	8	4	2
2016-2017	RRNA	21.1	Non-agricultural	7	10	4
2016-2017	LSNA	20	Non-agricultural	8	7	1
2017	LLNA	N/A	Non-agricultural	8	8	0
2017	LNNA	10.7	Non-agricultural	7	2	1
2017	KEAG	30.7	Agricultural*	4	8	1
2017	BEAG	40.1	agricultural	5	0	0
2016-2017	CLAG	38.9	agricultural	9	11	3
2017	ELAG	81.3	agricultural	3	0	0
2016-2016	GUAG	91.5	agricultural	8	8	2
2016-2017	SFAG	61.3	agricultural	2	0	0
2017	NDAG	30.7	agricultural	3	0	0
2016	DRAG	31	agricultural	0	0	1

*organic farm

Table 2: Spiked individual samples used to create the calibration curve using blank Tree Swallow urine samples.

Year	Tube	Spike (ug ml-1)
2016	A	0
2016	B	0
2016	C	10 6CN
2016	D	10 2CTCA
2016	E	10 6CN + 10 2CTCA
2016	F	25 6CN
2016	G	25 2CTCA
2016	H	25 6CN + 25 2CTCA
2016	I	0.1 6CN
2016	J	0.1 2CTCA
2016	K	0.1 6CN + 0.1 2CTCA
2016	L	1 6CN
2016	M	1 2CTCA
2016	N	1 6CN + 1 2CTCA
2017	O	0 6CN
2017	P	0.02 6CN
2017	Q	0.5 6CN
2017	R	1 6CN
2017	S	2 6CN
2017	T	0 6CN
2017	U	0.02 6CN
2017	V	0.5 6CN
2017	W	1 6CN
2017	X	2 6CN

Table 3: Neonicotinoid metabolites detected in individual insect samples taken from non-agricultural and agricultural nests in Guelph, ON for 2016 (n= 16 samples) and 2017 (n= 8 samples).

Year	Nest ID	Sample Weight (g)	Analyte		
			Imidacloprid	2CTCA	6CN
Non-agricultural					
2016	ASNA1	1.60			
2016	ASNA2	2.04			
2016	ASNA5	1.96			
2016	ASNA6	1.75		<MDL	
2016	GLNA2	1.96			
2016	GLNA6	1.97			
2016	HCNA3	2.02			
2016	HCNA5	2.01			
2016	RRNA2	2.00			
2016	RRNA3	1.97			
2017	LSNA5	1.99			
2017	RRNA3	2.05			
2017	RRNA3-2	1.99			
2017	GLNA3	2.02			
2017	ASNA7	2.04			
2017	LNNA	2.05			
Agricultural					
2016	GUAG3	0.84			
2016	GUAG5	2.05		<MQL (est = 0.0024 ppm)	
2016	CLAG2	2.01			
2016	CLAG3	1.95		<MDL	
2016	DRAG1	1.97			
2016	STAG	1.96		<MDL	
2017	KEAG4	2.01			
2017	CLAG2	1.97			

Table 4: Means of Imidacloprid present in Tree Swallow pooled nestling urine samples collected at Long Point Bird Observatory for the control (n= 24 samples), low (n= 28 samples) and high (n= 24 samples) treatments. Means are approximate to 3-digit scientific notation with standard error of the means (±SEM).

Treatment	**Time (hrs)**	**Mean IMI (ppm)**
Control	0	0
Control	3	$1.25 \times 10^{-4} \pm 1.25 \times 10^{-4}$
Control	6	0
Low	0	0
Low	3	$3.56 \times 10^{-4} \pm 1.83 \times 10^{-4}$
Low	6	$5.60 \times 10^{-4} \pm 3.67 \times 10^{-4}$
High	0	0
High	3	$9.67 \times 10^{-4} \pm 1.83 \times 10^{-4}$
High	6	$1.18 \times 10^{-3} \pm 3.84 \times 10^{-4}$

6. Appendix

Table 5: Raw data of pooled samples from TRES nestling dosing experiment at LPBO along with calculated z-score. Absent values indicate that no residues were found in the sample. Z-scores with a value >|2| were considered outliers and were not used in statistical analysis.

Treatment	Nest	Time (Hrs)	IMI (ug ml-1)	Z-Score
Low	80	0	0.0061	2.026683017
		6	<LOQ(0.0009)	0.02131214
	18	0	<LOD	-0.325771281
		3	<LOD	-0.325771281
		6	<LOD	-0.325771281
	2	0	<LOD	-0.325771281
		3	<LOD	-0.325771281
		6	<LOD	-0.325771281
	79	0		-0.325771281
		3	<LOQ(0.0021)	0.484090035
		6	<LOD	-0.325771281
	78	0	<LOD	-0.325771281
		3	<LOQ(0.0011)	0.098441789
		6		-0.325771281
	24	0	<LOD	-0.325771281

		3	<LOD	-0.325771281
		6	0.0031	0.86973828
	30	0	<LOD	-0.325771281
		3	<LOD	-0.325771281
		6	<LOQ(0.0016)	0.291265912
	28	0	<LOD	-0.325771281
		3	<LOD	-0.325771281
		6	<LOD	-0.325771281
	67	3		-0.325771281
		6	<LOD	-0.325771281
	44	0	<LOD	-0.325771281
		3		-0.325771281
		6		-0.325771281
High	7	0		-0.325771281
		3	<MDL	-0.325771281
		6	0.0036	1.062562403
	10	0		-0.325771281
		3	<MDL	-0.325771281
		6	<MDL	-0.325771281
	70	0	<MDL	-0.325771281
		3		-0.325771281

		6	<MQL (0.0011)	0.098441789
	19	0	0.019	7.001545387
		3	0.0037	1.101127228
		6	<MQL (0.0011)	0.098441789
	14	3	<MQL (0.0012)	0.137006614
		6	<MQL (0.0008)	-0.017252685
	50	0		-0.325771281
	34	3	<MQL (0.0009)	0.02131214
		6	<MQL (0.0015)	0.252701087
	38	0	<MDL	-0.325771281
		3	<MQL (0.0008)	-0.017252685
		6	<MQL (0.0008)	-0.017252685
	11	0		-0.325771281
		3	<MQL (0.0013)	0.175571438
		6	<MQL (0.0017)	0.329830736
	71	0	<MQL (0.0008)	-0.325771281
Control	73	3	<MDL	-0.017252685
	73	6	<MDL	-0.325771281
	15	0	<MDL	-0.325771281
		3	<MDL	-0.325771281
		6	<MDL	-0.325771281

27	0	<MDL	-0.325771281
	3	<MDL	-0.325771281
	6	<MDL	-0.325771281
35	0		-0.325771281
	3		-0.325771281
41	3	0.010	3.530711176
	6		-0.325771281
12	0		-0.325771281
	3	<MQL (0.0010)	0.059876964
	6	<MDL	-0.325771281
21	0	<MDL	-0.325771281
	3	<MDL	-0.325771281
62	0	<MDL	-0.325771281
	3	<MDL	-0.325771281
	6	<MDL	-0.325771281
65	3		-0.325771281
36	0	<MDL	-0.325771281
	3	<MDL	-0.325771281
	6	<MDL	-0.325771281